Reactivity of Synthetic Peptides Representing Selected Sections of Hepatitis C Virus Core and Envelope Proteins With a Panel of Hepatitis C Virus–Seropositive Human Plasma

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A series of 54 synthetic peptides, 15–20 residues long, that represented selected parts of the structural proteins of hepatitis C virus (HCV) were tested for immunoreactivity with a panel of 45 plasma samples from potential blood donors who were known to be seropositive for anti-HCV. Most of the ten peptides that represented the core protein showed reactivity with most of the panel samples. All except one of the 20 peptides that represented non-hypervariable regions of envelope proteins E1 and E2 showed little or no reactivity. In contrast, 18 of the the 24 peptides that represented variants of the hypervariable region 1 of the E2 protein reacted with at least one panel sample. Notably, 40% of the panel samples cross-reacted with two or more different peptides sequences some of which differed by more than 50%. Two panel samples each cross-reacted with seven different peptide sequences. The results suggest a broad anti-hypervariable region antibody specificity in many anti-HCV–seropositive samples and possible limits on the mutability of hypervariable region sequences. The work contributes to understanding the immunogenicity and persistence of HCV. ***J Med Virol 51:67–79, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Hepatitis C virus (HCV) is the aetiological agent responsible for more than 90% of parenterally transmitted liver disease formerly called non-A non-B hepatitis. [Choo et al., 1989, 1991]. Hepatitis C is relatively common with detectable HCV antibodies in approximately 0.05–1.5% of current blood donor populations. The disease is usually asymptomatic in the early stages but often progresses to a chronic infection and can result in hepatocellular carcinoma [Hopf et al., 1990; Choo et al., 1991; Esteban et al., 1991]. The viral genome is a single-stranded positive polarity RNA virus 9.5 kb long that codes for a polyprotein of approximately 3,000 amino acid residues that includes three structural proteins, core and envelope glycoproteins E1 and E2, and at least nine non-structural proteins that mediate replication [Choo et al., 1991; Lin et al., 1994]. It has six major genotypes and at least 11 subtypes [Chan et al., 1992; Simmonds et al., 1994]. In addition, the E2 protein has two short lengths of high sequence variability, hypervariable regions (HVR) 1 and 2 [Hijikata et al., 1991; Kato et al., 1991]. Isolates of HCV show markedly different sequences in these regions, and single samples often contain multiple closely related variants or quasi-species which may alter in a single patient during the course of the infection [Kato et al., 1991, 1993; Martell et al., 1992; Okamoto et al. 1992; Weiner et al., 1992; Higashi et al., 1993; Lesniewski et al., 1993; Taniguchi et al., 1993; Van Doorn et al., 1995]. The core and several non-structural proteins are strongly immunogenic [Chien et al., 1992; Sallberg et al., 1992], and the detection of the corresponding antibodies is the basis of current clinical tests for the infection [Kuo et al., 1989; Van der Poel et al., 1991]. Antibodies to the HCV envelope proteins are found in 95–100% of infected individuals but they appear to be often non-neutralising [Prince et al., 1992; Chien et al., 1993]. However, when a strong immune response to peptides that represent HVR sequences was elicited in chimpanzees there was a tendency to recovery, and low levels of response tended to lead to the chronic disease [Van Doorn et al., 1995]. The C-terminal half of HVR 1, residues 14–27, is immuno-

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genic [Kato et al., 1993], and an antibody response to this region correlates with a decrease or disappearance of the corresponding quasispecies which suggests that some neutralising antibodies may arise to that part of HVR 1 [Weiner et al., 1992; Kato et al., 1993; Taniguchi et al., 1993]. It has also been shown that antibodies that recognise expressed recombinant HVR 1 sequences can block viral attachment to cells [Zibert et al., 1995], and it has been proposed that the HVR 1 region is exposed and forms B-cell epitopes that are subject to immune selection. Escape from immune surveillance by rapid mutation that generates multiple quasispecies may enable the disease to become persistent in individuals [Kato et al., 1992a,b, 1993; Weiner et al., 1992; Taniguchi et al., 1993; Kato et al., 1994; Sekiya et al., 1994; Shimizu et al., 1994; Van Doorn et al., 1995]. By comparison, it is of interest that the hypervariable V3 loop of HIV 1 is the primary neutralising epitope [La-Rosa et al., 1990; Wolfs et al., 1990].

Synthetic peptides have become an important tool for the investigation of viral immunity and its detection. We have used this technology to measure and compare the immunoreactivity of a group of peptides, some of which represented the most hydrophilic parts of the core, E1, and E2 proteins and others which represented peptides derived from the carboxy-terminal half of HVR 1, using a panel of 45 plasma samples from HCV-infected potential blood donors who presented at the East Anglia Blood Centre. The data reported here show that an unexpectedly large proportion of HCV-infected plasma samples reacted with individual synthetic peptides that represented a range of HVR 1 sequences. The work is relevant to understanding the immunogenicity and persistence of HCV and has implications for the design of peptide vaccines.

MATERIALS AND METHODS

Plasma Samples

Samples of HCV-infected serum or plasma were obtained from 45 prospective blood donors presenting at the East Anglia Blood Centre, Cambridge, UK. HCV infection was shown by the presence of antibodies using an HCV screening test kit (HCV-EIA, Abbott Laboratories) and confirmed using an Ortho RIBA-2 test. Plasma from HCV-seronegative blood donors was used as controls.

Peptides

The polyprotein of a type 1b isolate of HCV (HCVJK1G), which is relatively common in the UK, and for which the full-length gene sequence was published (direct submission, Genbank division VRL, accession number 1596), was selected for analysis. The putative core and envelope proteins E1 and E2 were analysed to predict the most hydrophilic and, therefore, potentially the most immunogenic sections of the sequences by the method of Kyte and Doolittle [1982] with an analysis window of 18 residues, using the MacVector programme (IBI, Inc., New Haven, CT). A series of peptides ranging in size from 15 to 20 residues that represented the most hydrophilic and some non-hydrophilic sections were synthesised commercially by either Cambridge Research Biochemicals Ltd. (CRB) (Northwich, UK) or by Severn Biotech Ltd (SB) (Kidderminster, UK). Peptide E2.1 was the gift of Dr. B.E. Clarke (Wellcome Research Laboratories, Beckenham, Kent) (Table I). A series of peptides that represented HVR 1 were also obtained from these suppliers. Peptides were obtained at a purity of either greater than 70% (CRB) or 85% (SB and E2.1) and used without further purification. Peptides were obtained in freeze-dried form and were dissolved at a concentration of 1 mg/ml in dimethyl sulphoxide/H_2O (1:24, v/v) and stored frozen. HVR 2 was not investigated in this study.

Peptides HV1–9 corresponded to residues 14–28 of HVR 1 sequences from HCV isolates of genotypes 1a, 1b, and 2a for which the complete genome sequence has been published. Peptide HV1 was selected as it occurred in the original HCV sequence. Peptide HV2 was derived from variant HCVJK1G (see above) from which all the non-HVR peptides were derived. Peptides HV8 and 9 were included as they has been reported previously to contain an immunoreactive epitope [Kato et al., 1994]. Other published peptides (HV3–7) were chosen to maximise sequence differences from each other. To each of the peptide sequences HV1–9 was added either an N- or C-terminal cysteine to facilitate coupling to the bovine serum albumin (BSA) carrier (see Table II).

Peptides HV10–17 were de novo sequences derived, by alteration of specific residues, from two published HVR variants, HCV-1A and HCV-JH. Peptides HV10 and 17 were derived from variant HCV-1A. Peptide HV10 is identical with the corresponding sequence except for residue positions 3 and 7, and peptide HV17 is a modification of HV10 at positions 4, 8, and 12. Peptides HV11–16 were derived from HCV variant HCV-JH. Peptide HV11 is identical with the corresponding sequence from HCV-JH except for the two C-terminal residues (KI in HCV-JH). Peptides HV12–14 are variants of HV11 in which residue positions 1–5, 10, and 13–15 have been left unaltered. At the remaining positions various changes have been made to test the effects of either altered conformation (by introducing or omitting proline), or charge, or hydophilicity. Peptide HV15 is a combination of the N-terminal six residues of HV11 and the C-terminal nine residues of HV1. Peptide HV16 is identical with HV15 except that it has an additional N-terminal cysteine. Peptides HV18–24 corresponded to peptides HV10–17 (except HV16) but were synthesised with an additional C-terminal cysteine. All the HVR peptide sequences contained the conserved glycine and glutamine residues at positions 23 and 26, respectively, and proline, when included, was allowed only at positions 22 and 24 as it is found in published HVR sequences.

Coupling of the Peptides to BSA

Peptides that contained cysteine were cross-linked to BSA (Sigma Chemical Co., Poole, UK, A7030) using the heterobifunctional cross-linker 3-maleimidobenzoic

TABLE I. Peptides From the Core, E1, and E2 (Non-Hypervariable Region) Proteins*

Peptides	Sequence	Position	No. of reactive samples	Percent reactive samples	Hydrophilicity
Core protein					
C1	MSTNPKPQRKTKRNTNRR**C**	1–18 +C	37	82.2	+
C2	KTKRNTNRRPQDVKF**C**	10–24 +C	32	71.1	+
C3	GQIVGGVYLLPRRGP**C**	28–42 +C	33	73.3	−
C4	GPRLGVRATRKTSERSQP**C**	41–57 +C	18	40.0	+
C5	ATRKTSERSQPRGRRQPI**C**	48–65 +C	17	37.8	+
C6	PKARQPEGRAWAQPG**C**	66–80 +C	7	15.6	+
C7	GNEGLGWAGWLLSPY**C**	87–101 +C	NIL	NIL	+
C8	SRPSWGPTDPRRRSRNLGC	103–120 +C	3	6.7	+
C9	LMGYIPLVGAPLGGAARA**C**	133–150 +C	NIL	NIL	−
C10	TGNLPG**C**SFSIFLLALLS**C**	166–183 +C	NIL	NIL	−
E1 protein					
E1.1	YEVRNVSGVYHVTND**C**SN**C**	1–18 +C	1	2.2	+
E1.2	GVYHVTND**C**SNSSIVYEA**C**	8–25 +C	NIL	NIL	+/−
E1.3	AADMIMHTPG**C**VP**C**V	25–39	NIL	NIL	−
E1.4	SR**C**WVALTPTLAARN	45–59	NIL	NIL	−
E1.5	AARNSSIPTTTIRRHVDL**C**	56–73 +C	NIL	NIL	−
E1.6	VGAAAL**C**SAMYVGDL**C**GS**C**	75–92 +C	NIL	NIL	−
E1.7	SPRRYETVQD**C**N**C**SLYPG**C**	103–120 +C	NIL	NIL	+
E1.8	SLYPGHVSGHRMAWDMMM**C**	116–133 +C	1	2.2	+
E1.9	**Y**PGHVSGHRMAWDMM	118–132	1	2.2	+
E1.10	SGHRMAWDMMMNWSP**C**	123–137 +C	19	42.2	+
E1.11	**A**WDMMMNWSPTTALV	128–142	1	2.2	−
E1.12	PQAVVDMVVGAHWGVLAG**C**	150–167 +C	NIL	NIL	−
E2 protein					
E2.1	QLVNTNGSWHINRTALN**C**	29–46	4	8.9	+
E2.2	RPIDRFAQGWGPITH**C**	77–91 +C	NIL	NIL	+
E2.3	GWGPITHAESRSSDQRPY**C**	85–102 +C	NIL	NIL	+
E2.4	LQVCGPVY**C**FTPSPVVVG**C**	117–134 +C	NIL	NIL	−
E2.5	NETDVLLLNNTRPPQ**C**	149–163 +C	NIL	NIL	+
E2.6	DCFRKHPEATYTKCGSGP**C**	201–218 +C	NIL	NIL	+
E2.7	**V**EHRLNAACNWTRGE	253–267	NIL	NIL	+
E2.8	GVGSAVVSIVIKWEYVLL**C**	321–388 +C	NIL	NIL	−

*The synthetic peptide sequences shown are all from HCV isolate HCVJK1G and were chosen to represent mostly the most hydrophilic sections the proteins with some hydrophobic sections as controls. The coupling of each peptide to BSA was through the residues indicated in bold face type. The peptide position indicates the peptide residues in each relevant structural protein when numbered separately from their amino termini. The number of reactive plasma samples that reacted with each peptide was abstracted from Table III and is expressed as a percentage of the total number of plasma samples (45). The hydrophilicity was determined as described in Materials and Methods.

acid N-hydroxysuccinimide ester (MBS) (Sigma Chemical Co., M2786). In a typical experiment the BSA was activated as follows: To 2.5 ml of BSA solution (10 mg/ml in phosphate-buffered saline) was added 1.0 ml of MBS solution (10 mg/ml in dimethylformamide, made immediately before use) and allowed to react at room temperature (approx. 22°C) for 30 min. The MBS-activated BSA was separated from residual MBS by gel filtration on a Sephadex G50 (medium grade) (Pharmacia-LKB Ltd., Milton-Keynes UK) column (16 mm × 250 mm) eluted in 50 mM sodium phosphate buffer pH 6.0 at 22°C with a flow rate of 25 ml/hr. The eluent absorbance was measured at 280 nm, and the MBS-activated BSA corresponded with the first absorbance peak which was collected and diluted with eluent buffer to 2.5 mg/ml BSA, assuming that all the BSA was recovered. To couple each peptide, 1 ml of MBS-activated BSA solution, equivalent to 2.5 mg of BSA, was taken, and to it was added 1.5 ml of 50 mM sodium phosphate buffer pH 6.0, 0.5 ml of a solution of the peptide (0.5 mg) to be coupled, and 3.5 ml of 50 mM di-sodium hydrogen phosphate (without pH adjustment) to raise the pH to approximately 7.3. The mixture was incubated at 22°C for 3 hr and then dialysed exhaustively against PBS at 4°C. The volume of the dialysed peptide-BSA conjugate was adjusted with PBS to a final BSA concentration of 250 μg/ml and then stored frozen.

Peptides that did not contain cysteine were treated with 2-iminothiolane hydrochloride (ITL) (Sigma Chemical Co., I 6256) so as to add a small linker arm, having a sulphydryl function, to any amino groups that occurred either as part of any lysine side chain or at the amino terminus. In a typical experiment, to 0.5 ml of a solution of peptide (0.5 mg) was added 0.5 ml of 50 mm sodium borate buffer pH 9.0, 10 μl of 0.1 M ethylenediaminetetraacetic acid di-sodium salt ($EDTANa_2$), and, for each amino-group present in each peptide, one aliquot of 50 μl of 0.5 M ITL solution in H_2O. The mixture was incubated at 22°C for 2 hr and then applied to a Sephadex G10 column (16 mm × 250 mm) and eluted in 50 mM sodium phosphate buffer pH 6.0, 1 mM $EDTANa_2$ at 22°C with flow rate of 25 ml/hr. The eluent absorbance was measured at either 280

TABLE II. Peptides From the Hypervariable Region 1 of E2 Protein*

Peptide	Sequence	Genotype	No. of reactive samples	Percent reactive samples	Peptide	Sequence	Genotype	No. of reactive samples	Percent reactive samples
A					B				
HV1	**C**SGFVSLLAPGAKQNV	1a	nil	nil					
HV2	**C**RRVASFFSPGSAQKI	1b	7	15.6	HV3	RRVASFFSPGSAQKI**C**	1b	9	20.0
HV4	**C**LGIASFLTRGPKQNI	1a	3	6.7	HV5	LGIASFLTRGPKQNI**C**	1a	5	11.1
HV6	**C**RTLTGMFSLGARQKI	2a	7	15.6	HV7	RTLTGMFSLGARQKI**C**	2a	7	15.6
HV8	**C**RGFTSLFSAGSAQN	1b	1	2.2	HV9	RGFTSLFSAGSAQN**C**	1b	3	6.7
C					D				
HV10	**S**GLVSLFAPGA**K**QNV	—	9	20.0	HV18	SGLVSLFAPGAKQNV**C**	—	3	6.7
HV11	**S**TLTSLFRPGASQNV	r1b	3	6.7	HV19	STLTSLFRPGASQNV**C**	r1b	5	11.1
HV12	**S**TLTSLFTPGPAQNV	—	2	4.4	HV20	STLTSLFTPGPAQNV**C**	—	nil	nil
HV13	**S**TLTSLFSPGPSQNV	r1b	6	13.3	HV21	STLTSLFSPGPSQNV**C**	r1b	nil	nil
HV14	**S**TLTSLFTPGASQNV	r1b	7	15.6	HV22	STLTSLFTPGASQNV**C**	r1b	nil	nil
HV15	**S**TFTSLLAPGA**K**QNV	—	4	8.9	HV23	STFTSLLAPGAKQNV**C**	—	nil	nil
HV16	**C**STFTSLLAPGAKQNV	—	14	31.1					
HV17	**S**GLTSLFTPGASQNV	—	8	17.8	HV24	SGLTSLFTPGASQNV**C**	—	nil	nil

*The synthetic peptide sequences shown are all derived from residues 14–28 of the HVR 1 of HCV isolates. A: peptides HV1, 2, 4, 6, and 8 were from different published HCV isolates and were coupled to BSA through their aminoterminus or lysine: B: published peptides shown in A but coupled through their carboxy-terminal cysteine. **C:** peptides 10 and 17 were altered versions of the hypervariable region of HCV isolate HCV-1A. Peptide HV11 is identical with the corresponding section of HCV-JH except for the final two residues (KI in HCV-JH). Peptides HV12–16 are altered versions of peptide HV11. Peptides HV10–17 were coupled to BSA through their amino-terminal residue or lysine: D: peptides as shown in C but coupled through their carboxy-terminal cysteine. The coupling of each peptide to BSA was through the residues indicated in bold face type. The various changes made to the peptide sequences are described more fully in the text. The number of plasma samples that reacted with each peptide was abstracted from Table IV and is expressed as a percentage of the total number of plasma samples (45). The subtypes shown were deduced from the complete E1 and E2 protein sequences in which the relevant HVR peptides occurred. HV11 was virtually identical with isolate HCV-JH (subtype 1b). It therefore resembles subtype 1b and so has been denoted as subtype r1b for the purposes of the present work. Peptides HV13 and 14 differ from peptides HV11 only in 2 and 1 residues, respectively, and have therefore also been denoted as subtype r1b. The genotype nomenclature of Simmonds et al. [1994] is used.

nm or 215 nm (depending on whether or not the peptide absorbed at 280 nm). The ITL-reacted peptide corresponded with the first peak of absorbance in the eluent, which was collected in 5 ml and to this solution was added 1 ml of a solution of MBS-activated BSA (2.5 mg), prepared as described previously, and 1.5 ml of 0.5 M di-sodium hydrogen phosphate to bring the pH to approximately 7.3. The mixture was incubated at 22°C for 3h and then dialysed exhaustively against PBS at 4°C. The volume of the dialysed peptide–BSA conjugate solution was adjusted with PBS so that the final BSA concentration was 250 μg/ml and then stored frozen.

Immunoassay

The reactivity of the various peptides with the panel of infected sera was determined using a enzyme-linked immunosorbent assay (ELISA) as follows: The wells of a 96-well microtitre plate (Nunc-Immuno plate, Maxisorp; Life Technologies, Glasgow, Scotland) were coated using 100 μl per well of a solution of peptide-BSA conjugate (2.0 μg BSA/ml in PBS) and incubated at 4°C for 16 hr. The wells were washed 3 times with H_2O and incubated for 1 hr at room temperature (22°C) with BSA solution (2.0 mg/ml in H_2O) in order to prevent non-specific absorption. The wells were incubated for 1 hr at 40°C with the plasma samples diluted 100-fold in sample buffer (see below) using 180 μl/well and subsequently for 1 hr at 40°C with a solution of goat anti-human IgG serum conjugated to peroxidase used at a 14-fold dilution in conjugate buffer (see below) (190 ml/well). After each of the latter two incubation steps the wells were washed 3 times with 0.5% (v.v) Tween 20 (BDH, Poole, UK) solution. The peroxidase reaction was visualised using the o-phenylenediamine solution in substrate dilution buffer (see below) (200 μl/well). After 30 min incubation at 22°C the reaction was stopped by the addition of 50 μl of 4 M sulphuric acid to each well, and the absorbance was measured at 492 nm on a Titertech 96-well plate reader. The goat anti-IgG-peroxidase conjugate, the substrate, and the three dilution buffers, that is, for the serum, the conjugate and the substrate, were all obtained from an HCV screening EIA kit (Abbott Laboratories, N. Chicago, Illinois). The cut-off was determined after using the standard ELISA to measure the reactivity of 192 HCV-seronegative plasma with two representative reactive peptides, C1 (mean absorbance, 0.208) and HV16 (mean absorbance, 0.185). Four standard deviations included all except one negative sample for both peptides C1 and HV16. In addition, the mean of the 23 negative controls included in each plate was within 6% of the 192 negative population mean. The cut-off for each plate was calculated as the mean plus 4 times the standard deviation for the absorbancies of at least 20 anti-HCV–negative plasma that were included on each plate. Each plate was duplicated, and samples were considered to be positive when their absorbance was above the cut-off on both plates. When using this value for the cut-off no false positive results were obtained amongst any of the negative control plasma samples.

Virus Genomic Detection and Genotyping

Viral RNA was extracted from 100 μl of plasma samples using HCV specific biotinylated capture oligonucleotide and streptavidin-coated paramagnetic particles (Petrik, J., Pearson, G. and Allain, J.-P., manuscript accepted for publication). RNA was eluted from the washed particles in a small volume and used directly for the reverse transcription–polymerase chain reaction step (RT-PCR). RT-PCR was carried out as described previously [Cristiano et al., 1992] with the following modifications: The reaction volume was decreased to 20 μl for both PCR steps. After 30 min at 37°C 30 cycles were carried out each consisting of 95°C/20 sec, 48°C/25 sec, and 72°C/30 sec using a Perkin Elmer 9600 system. The first reaction solution (volume 1 μl) was used as template for 30 cycles of nested PCR with internal primers under the same conditions. The nested PCR products (5 μl) were analysed on 1.3% agarose gels. After electrophoresis the gel was denatured and neutralised using standard methods [Sambrook et al., 1989], blotted onto a nylon membrane (Boehringer Mannheim UK Ltd., Lewes, UK) using a positive pressure blotting system (PosiBlot, Stratagene, La Jolla, CA), UV-fixed, hybridised to a digoxygenin (DIG)-labelled oligonucleotide probe, washed, and detected using a DIG chemiluminescent kit (Boehringer Mannheim UK Ltd.) according to the manufacturer's instructions. Genotyping was carried out using a INNO-LiPA HCV kit (Innogenetics, Zwijndrecht, Belgium) according to the manufacturer's instructions.

RESULTS

The Reactivity of the Core, E1, and E2 Peptides

The amino acid sequences of the peptides that were tested are shown in Tables I and II together with their coupling position, hydrophilicity, genotypes where appropriate, and their frequency of reactivity with the panel of human HCV-positive donor plasma expressed as the percentage of the total number of plasma samples that reacted with any individual peptide (percent-reactivity). The frequency of reactivity showed a wide spread from 0 to 82.2% depending on the peptide tested. The reactivity data is shown in detail in Tables III and IV. The hydrophilicity profiles of E1 and E2 proteins, produced by the MacVector program, were similar irrespective of genotype (data not shown). The peptide hydrophilicity ratings shown in Table I were determined by inspection of the profiles. Peptides representing parts of the core protein, in particular those closest to the N terminus, had the highest percent-reactivity. Some of those core peptides representing sequences predicted to have high hydrophilicity did have the predicted higher levels of reactivity with the donor samples as shown by peptides C1, C2, C4, C5, and C6. Conversely, the hydrophobic peptides C9 and C10 had no reactivities with any sample. However, the correlation between the frequency of reactivity and hydrophilicity was imperfect since the slightly hydrophobic peptide C3 reacts with 33 (73.3%) of the panel of plasma samples and the

TABLE III. The Reactivity of the Plasma Samples (a) With the Synthetic Peptides Representing the Core, E1 and E2 (Non-Hypervariable Region) Proteins of HCV and the Corresponding Clinical Data*

Plasma sample No.	Peptides													Clinical data			
	CORE							E1					E2				
	C1	C2	C3	C4	C5	C6	C8	E1.1	E1.8	E1.9	E1.10	E1.11	E2.1	ALT[a]	RNA[b]	Genotype[c]	EIA[d]
1	4.3	3.1				1.4								R	+	1	6.05
2	4.4	3.5	4.2	1.3							2.7			R	+	1b	5.85
3	4.5	3.6	4.1								3.2			N	+	nd	5.12
4	4.4	3.6	3.4	4.6	3.9	3.2	2.3				1.4			N	+	nd	5.65
5	4.4	2.8	4.3								6.2		1.4	R	−	nd	5.65
6	4.2	3.5	3.8											R	−	nd	5.51
7	4.4	3.6	3.8	4.5	4.0	3.2								R	+	1a	5.45
8	4.2	3.5	4.0											R	+	1	5.44
9	4.4	3.7	4.2	4.2	1.5					1.8	4.4			N	+	1a	5.54
10	4.4	3.7	4.0		1.8						3.1	1.1		N	+	1b	4.88
11	4.4	1.3	3.4											R	+	nd	4.7
12	4.2	3.6	3.4	2.9	2.0	3.0								N	+	nd	4.34
13	4.6	3.7	4.3					1.1						N	+	1a	3.74
14	4.6	3.8	4.0	4.5		2.0					6.1		1.5	N	+	1	6.06
15	4.5	3.8	4.0	2.7	2.2									N	+	2b	5.41
16	4.6	3.8	3.4	4.6							1.2			N	+	2a	5
17	1.9		2.5		1.5									R	+	nd	4.3
18	4.0	3.0	1.4											R	+	1	5.71
19					1.4									N	+	3a	4.82
20	4.4	3.8												N	−	nd	6.52
21	4.6	3.8	4.4		2.0						3.4			N	−	nd	6.01
22	4.8			2.8										N	+	3a	6.27
23														N	+	1a	4.95
24	4.4		4.5	4.5	4.1						6.2			R	−	nd	6.02
25			4.3								3.2			N	+	1a/2	5.61
26	4.4	3.3	4.2	3.3	2.7						3.6			R	−	nd	5.57
27	2.3		4.0								3.1			N	+	1	4.73
28														N	−	nd	2.9
29	4.6	1.7	4.3	4.3	3.6									N	−	nd	5.96
30	2.6	1.4	3.6										1.2	N	+	3a	4.61
31	4.6	1.7	3.2	1.6	4.1		2.0							R	+	3a	5.48
32	2.7	2.0		1.0										R	+	1	3.73
33														N	−	nd	2.81
34	4.4	3.7	4.1	4.4	2.7	2.5					2.8			N	+	nd	5.33
35	4.4	3.0	4.1			1.1	1.2				1.4			N	+	1b	4.85
36	1.3	3.7												N	+	3	4.79
37	4.3	3.2	4.2	4.3	2.6									R	−	nd	3.21
38			4.2								2.4		2.3	R	+	1a	5.29
39	4.3	3.5												N	+	nd	6.32
40														N	+	1a	6.65
41	4.4	3.5	4.1	1.5	1.5						1.2			N	+	1	6.65
42			1.6											R	−	nd	5.29
43	4.4	3.2	3.4	1.9	2.1				2.3		5.3			N	+	nd	5.1
44	4.2	2.5	2.6								1.7			R	+	1b	5.01
45	3.0													N	+	1b	5.54
Total reactive samples																	
	37	32	33	18	17	7	3	1	1	1	19	1	4				

*All the assay procedures are described in Materials and Methods. The peptide sequences and their positions in the relevant proteins are shown in Table I. The mean for two measurements of the Abs/C-O ratios (cut-off = mean + 4 × SD) are shown for each positively reacting plasma sample (where both ratios were >1). Blank space indicates no reaction.
[a]R = raised alanylamino transferase (ALT) level; N = normal ALT level.
[b]+ = presence of RNA detected; − = RNA not detected.
[c]nd = genotype not determined. The nomenclature of Simmonds et al. [1994] is used.
[d]EIA refers to the clinical HCV screening assay. Values shown are the Abs/C-O ratios.

strongly hydrophilic peptide C8 reacted with only three panel members (6.7%). The core protein showed markedly higher levels of hydrophilicity than either of the putative envelope proteins, and the reactivity of the core protein peptides contrasted strongly with those from the E1 and E2 proteins (Tables I, III); not only do they have higher frequency of reactivity but their absorbance to cut-off ratios (Abs/C-O) are generally higher also. Peptide C1 had the highest frequency of reaction (82.2%) and a high level of reactivity (mean Abs/C-O, 4.1, range 1.3–4.8, median 4.4).

In E1 protein only one peptide, E1.10, had high frequency of reactivity (42.2%) and high levels of reaction (mean Abs/C-O 3.3). This high level of reactivity contrasts with that of the remaining E1 protein peptides of which only four were reactive, each with a single

different plasma sample. Peptides E1.8, E1.9, and E1.11 have sections of sequence in common with peptide E1.10. However, in peptide E1.10 the presence of both the N-terminal sequence SGHRM, which is present in peptides E1.8 and E1.9 but absent from E1.11, and the C-terminal sequence NSWP, which is present in E1.11 but absent from E1.8 and E1.9, appears to contribute to forming the reactive epitope (Table I).

The E2 protein has a noticeably higher proportion of its length that is hydrophilic and higher levels of hydrophilicity than the E1 protein. However, the peptides representing the non-HVR part of the E2 protein (residues 29 to 345) showed an almost complete lack of reactivity with the donor samples. Only peptide E2.1 had any reactivity at 8.9%.

HVR Derived Peptides

The differences in percent-reactivity between the HVR and non-HVR peptides of E2 protein is marked (Tables I–IV). Eighteen of the 24 HVR peptides were reactive. Both the natural peptides (HV1–9) and the de novo peptides, none of which were known to occur naturally, that were coupled to BSA at their N-terminal ends (peptides HV10–17) had a similar range of percent-reactivities (de novo 2.2–20% vs. natural 4.4–31.1%). The shorter peptides HV8 and HV9 which were described previously [Kato et al., 1994] have frequency of reactivities of 2.2 and 6.7%, respectively.

All of the natural HVR peptides (HV1–9), except HV1, had considerable levels of reaction (Abs/C-O: mean 2.4, range 1.0–4.7, median 2.4). The changes in percent-reactivity of the corresponding pairs of these peptides (HV2/3, HV4/5, HV6/7, and HV8/9), whether coupled at their N- or C-terminal ends, are unremarkable (Tables II, IV). The de novo peptides that were coupled to BSA at their N-terminal ends (peptides HV10 to 17) had similar levels of reaction to the natural peptides (Abs/C-O: mean 2.1, range 1.0–3.7, median 2.5). However, in contrast, the percent-reactivity of most of the de novo peptides, but not the natural peptides, were affected considerably by the position of the residue through which they were coupled to BSA. Thus, N-terminally coupled peptides HV12, 13, 14, 16, and 17 were reactive with numerous panel samples, but their corresponding C-terminally coupled peptides, HV20, 21, 22, 23, and 24, were all unreactive. Peptide HV16, which was coupled only through its N-terminal cysteine, had the highest percent-reactivity of any of the HVR peptides at 31.1%. Peptide pair HV11 and HV19 is the one exception in that the C-terminally coupled peptide has a similar percent-reactivity. These two peptides have a sequence which differs from that in a naturally occurring strain, HCV-JH, by solely the C-terminal residues which are KI in HCV-JH instead of NV.

Comparison of the frequency of reactivity of particular peptides provides evidence for the immunoreactive importance of certain residues. Thus, in the case of peptides HV16 and HV1 the change in the four N-terminal residues, STFT to SGFV, caused a decreased percent-reactivity from 31.1 to nil. Similarly, when peptide HV12 is changed from PA to AS at positions 24 and 25 of HVR 1 in peptide HV14 the percent-reactivity increased from 4.4 to 15.6. Peptide HV11 has one residue difference from HV14, R changed to T, at HVR 1 position 21; however, the latter has more than double the percent-reactivity, and only one of the plasma samples that reacted with peptide HV11 cross-reacted with peptide HV14. In contrast, substituting a T for a G at HVR 1 position 15 (peptides HV14 and 17) did not significantly alter the percent-reactivity, although not all of the reactive samples were the same for each peptide (see Table IV). The effect of a single residue change is also shown in the C-terminally coupled de novo peptides HV19 and 22 (R to T) causing a change from 11.1 to nil percent-reactivity.

Reactivity of the Plasma Samples

More than 93% of the plasma samples reacted with at least one of the core peptides and 68.9% reacted with at least three (see Table III). This contrasts strongly with the paucity of reactivity with the E1 protein peptides (except for peptide E1.10) where only four plasma samples reacted, each with a different peptide, and non-HVR E2 peptides where four plasma samples reacted but with only one peptide, E2.1. The low number of plasma samples that reacted with most of the E1 and E2 protein peptides is also striking in comparison with the much higher number, 29 of 45 (64.4%), that reacted with at least one HVR peptide. The level of reaction was highest with peptide C1 (Abs/C-O, mean 4.1, median 4.4), intermediate with peptide E1.10 (Abs/C-O, mean 3.3, median 2.5), and low with peptide HV16 (Abs/C-O, mean 2.35, median 2.4). Twenty-two (48.9%) of the plasma sample panel reacted with more than one HVR peptide (Table IV; Fig. 1). In some cases different plasma samples reacted with the same peptide, but each plasma sample that reacted with multiple HVR peptides did so with a different set of peptides. In a similar manner, for every peptide that was recognized by more than one plasma sample there was a different set of plasma samples which recognised it. The diversity of this reactivity is shown in Table V where the percentage of identical amino acid residue positions in the HVR peptide sequences and the numbers of plasma samples that have a cross-reaction with more than one peptide are shown comparatively. When the coupling position is ignored, all peptides had more cross-reactive samples than uniquely reactive ones, and five sequences had no unique reactors. Cross-reactivity was unrelated to peptide homology as it was more frequent with peptides less than 50% homologous, 56 from a total of 104 (Table V). The cross-reactivity has a notable diversity in that 14 plasma samples reacted with at least two peptides that differ in sequence by more than 60%. Two plasma samples cross-reacted with seven different sequences. The frequency of cross reactivity is indicated in Figure 1 where the percent of the total plasma samples that cross-reacted with a defined number of peptides is shown. There was no correlation between the genotype

TABLE IV. The Reactivity of the Plasma Samples With the Hypervariable Region 1 Peptides*,[a]

Plasma sample No.	Peptide[a]	Known HVR 1 peptides							De novo peptides related to HVR 1				
		HV1 (C)	HV2 (C)	HV3 (C)	HV4 (C)	HV5 (C)	HV6 (C)	HV7 (C)	HV8 (C)	HV9 (C)	HV10 (N)	HV11 (N)	HV12 (N)
1													
2													
3						2.5							
4			3.0	4.7			3.9	3.8			3.1		
5													
6						3.2							
7													
8			2.9	1.5							2.3		1.7
9												1.3	2.1
10			1.6	1.2									
11													
12													
13													
14							2.0	1.3					
15			2.0	2.1									
16			2.4	3.0							3.2		
17									2.2	2.9	2.8		
18													
19							2.5						
20													
21			2.8	2.3	1.4	1.3	4.0	3.5			3.1		
22													
23													
24										3.2			
25							3.8	3.6					
26											2.9		
27				1.2			1.4	1.1			2.8		
28													
29													
30													
31								1.2			3.0	2.0	
32													
33													
34				1.0							1.5	1.2	
35			1.2	1.8			2.6	2.0	1.1	1.8			
36													
37													
38					3.7	3.7							
39													
40													
41					3.8	3.0							
42													
43													
44													
45													
Total reactive samples		NIL	7	9	3	5	7	7	1	3	9	3	2

continued

of the sequence from which the HVR peptide was taken and the cross-reactivity with peptides of similar genotype origin.

Further analysis shows that 19 of the 45 plasma samples reacted with at least one of the published HVR peptides (HV1-9), and a total of 16 plasma samples reacted with just three of these peptides, HV3, 5, and 6 (Table IV), which are considerably different, having sequence homologies in the range 20–53%. In the case of the de novo peptides an even greater total reactivity was seen in which 24 samples were reactive and 20 reacted with just two specific peptides, HV14 and 16 that were 73% similar.

Seven plasma samples reacted with one or no peptides from the core, E1, and E2 proteins (Table III); they also reacted poorly with the HVR peptides (one or no reaction). In contrast, nine other panel members which did not react with HVR peptides reacted with two to seven core, E1, or E2 protein peptides. For any particular donor no correlation was found between the numbers of core, E1, and non-HVR E2 protein peptides and the numbers of HVR peptides that reacted. For each HVR peptide the mean Abs/C-O for every positive reaction was calculated and compared with the corresponding percent-reactivity, but no correlation was found.

TABLE IV. Continued

Plasma sample No.	De novo peptides related to HVR 1												Total reactive peptides	Different sequences recognised[b]
	HV13 (N)	HV14 (N)	HV15 (N)	HV16 (N)	HV17 (N)	HV18 (C)	HV19 (C)	HV20 (C)	HV21 (C)	HV22 (C)	HV23 (C)	HV24 (C)		
1													NIL	NIL
2	1.5	1.5					1.0						3	3
3			2.3	2.8									3	2
4				2.6		1.2							7	4
5													NIL	NIL
6				3.2									2	2
7	1.3												1	1
8				1.5	2.6								6	5
9				2.3			1.7						4	3
10													2	1
11	1.2			3.1									2	2
12													NIL	NIL
13				1.9									1	1
14	3.0		1.7	2.8									5	3
15	1.3												3	2
16		2.0			2.9	1.2							6	4
17				1.1	1.1								5	4
18													NIL	NIL
19													1	1
20													NIL	NIL
21		3.1	2.9		3.6								10	7
22				3.0									1	1
23													NIL	NIL
24													1	1
25		1.3			2.2								4	3
26						2.2							2	1
27		1.8	1.5	3.1	1.3		1.1						9	7
28				2.5									1	1
29													NIL	NIL
30													NIL	NIL
31		2.5			2.1		3.7						6	5
32													NIL	NIL
33													NIL	NIL
34					1.0		2.3						5	4
35				1.9									7	4
36	1.9	1.8											2	2
37													NIL	NIL
38													2	1
39													NIL	NIL
40													NIL	NIL
41													2	1
42				1.1									1	1
43													NIL	NIL
44													NIL	NIL
45													NIL	NIL
Total reactive samples	6	7	4	14	8	3	5	NIL	NIL	NIL	NIL	NIL		

*All the assay procedures are described in Materials and Methods. The mean for two measurements of the Abs/C-O ratios (cut-off = mean + 4 × SD) are shown for each positively reacting plasma sample (where both ratios were >1). Blank space indicates negative reaction.

[a]The peptide sequences are shown in Table II. Peptides were coupled through either the amino-terminal or lysine (N) or through cysteine (C).

[b]The number of different peptide sequences that react with each plasma sample is shown. Peptides within the following reactive pairs had the same sequence and were counted as a single reactivity: HV2 and 3, HV4 and 5, HV6 and 7, HV8 and 9, HV10 and 18, HV11 and 19, HV15 and 16.

Clinical Data

The clinical data for the donor samples are shown in Table III. No correlation was found between any of the measured parameters, that is, the presence of detectable viral genome by PCR, the genotype, the ALT level, the Abs/C-O in the clinical screening assay (marked EIA in Table III), and the number of different peptides with which each donor sample reacted whether from the core, E1, or E2 proteins including the HVR. Similarly the number of reactive bands on the RIBA-2 test showed no correlation with percent-reactivity (data not shown).

DISCUSSION

There is now a considerable body of work that documents the variability and immunogenicity of the HVR

TABLE V. The Percentage Homology of the HVR1 Peptide Sequences and the Number of Cross-Reactive Samples*

Peptide sequence	Peptide genotype[a]	Peptide sequence 1	2 + 3	4 + 5	6 + 7	8 + 9	10 + 18	11 + 19	12 + 20	13 + 21	14 + 22	15 + 16 + 23	17 + 24	Cross reactors[b]	Unique reactors[c]
1	1a	100 (nil)	27 (nil)	47 (nil)	20 (nil)	40 (nil)	80 (nil)	60 (nil)	53 (nil)	53 (nil)	60 (nil)	80 (nil)	67 (nil)	nil	nil
2 + 3	1b		100 (9)	33 (1)	47 (4)	53 (1)	33 (5)	33 (1)	40 (1)	40 (1)	33 (3)	27 (5)	27 (5)	8	1
4 + 5	1a			100 (5)	20 (1)	33 (nil)	33 (1)	27 (nil)	40 (nil)	33 (nil)	33 (1)	40 (3)	40 (1)	3	2
6 + 7	2a				100 (8)	53 (1)	40 (3)	47 (2)	40 (nil)	40 (1)	40 (4)	33 (5)	33 (4)	7	1
8 + 9	1b					100 (3)	40 (1)	47 (nil)	53 (nil)	53 (nil)	47 (nil)	43 (2)	43 (1)	2	1
10 + 18							100 (9)	73 (2)	67 (1)	67 (nil)	73 (4)	73 (5)	80 (7)	8	1
11 + 19	r1b							100 (3)	80 (1)	87 (1)	93 (3)	73 (2)	87 (2)	5	nil
12 + 20									100 (2)	87 (nil)	87 (nil)	67 (2)	80 (1)	2	nil
13 + 21	r1b									100 (6)	87 (2)	67 (2)	73 (nil)	5	1
14 + 22	r1b										100 (7)	73 (2)	87 (5)	7	nil
15 + 16 + 23												100 (15)	67 (4)	11	4
17 + 24													100 (8)	8	nil

*The first number in each box is the percentage homology for each pair of peptide sequences compared followed, in parentheses, by the number of plasma samples that cross-reacted with each peptide sequence. The data were taken from Table IV. The coupling positions of the peptides have been disregarded, and the data for identical sequences have been aggregated.
[a]The genotypes shown were deduced from the complete E1 and E2 protein sequences in which the relevant peptides occured. The nomenclature of Simmonds et al. [1994] is used. HV11 differs from the corresponding region of strain HCV-JH (genotype 1b) only in its C-terminal residue. It therefore resembles subtype 1b and has been denoted as genotype r1b for the purposes of the present work. Peptides HV13 and 14 differ from peptides HV11 only in 2 and 1 residues, respectively, and have therefore also been denoted as genotype r1b.
[b]The total number of plasma samples that cross-reacted with each peptide sequence (irrespective of the coupling position).
[c]The number of plasma samples that reacted solely with the indicated sequence.

Fig. 1. The distribution of the plasma sample reactivity and cross-reactivity with HVR 1 peptides. The numbers of plasma samples that react with the defined number of HVR peptides shown (of any sequence tested) are expressed as a percentage of the total number of plasma samples tested (45). The peptide numbers include both the published and the de novo HVR sequences.

of HCV. We have extended this data by testing the immunoreactivity of a number of synthetic peptides derived from both the HVR and the non-HVR part of the structural proteins against a panel of 45 seropositive plasma samples. Our data confirm earlier work in which peptides that represented the amino terminal region of the core protein have been shown to be recognised by most patients' antibodies [Sallberg et al., 1992], whereas those of E1 and the non-HVR of E2 proteins are commonly unreactive [Zhang et al., 1994] (Table III). The levels of the Abs/C-O ratios that we determined correlate with this pattern, the mean reactivities for all of the positively reactive plasma samples for the core protein (3.4) being higher than those for E1 and E2 proteins (3.0 and 1.6, respectively). The data are also consistent with the work of Ching et al. [1992] in that the peptide immunoreactivity does not always reflect the hydrophilicity predictions. However, the peptides that we tested were not glycosylated, which is known to increase antibody recognition of recombinant forms of E1 and E2 protiens [Lok et al., 1993]; peptides E1.1, E1.2, E1.5, E1.7, E1.10, E1.11, E2.1, and E2.5 all contained potential glycosylation sites, but all except E1.10 had low or zero reactivity. The peptides were relatively short and therefore we would not have expected to detect conformational epitopes However, comparison of the reactivity peptide E1.10, which is strongly immunogenic, as indicated in the previous the work of Ray et al. [1994], with that of the overlapping peptides, E1.8, E1.9, and E1.11, indicated the apparent cooperative effect of the non-continuous sequences SGHRM and MNSWP in forming the epitope.

Our data demonstrate clearly the importance of specific HVR residue positions for epitope formation, a phenomenon that has been observed by other workers using other HVR-derived peptides [Taniguchi et al., 1993; Sekiya et al., 1994; Kato et al., 1994]. Comparison (Table II) of the percent-reactivity of both the N-terminally coupled de novo peptides HV11, 12, 14, and 17 and the

C-terminally coupled de novo peptides HV19 and 21 shows the marked effects of specific residue changes. These results highlight our finding that numerous plasma samples each recognise several very different peptide sequences. They also appear to complement the observation, from analysis of several hundred HVR sequences in the Genbank database, and which has been referred to previously [Lesniewski et al., 1993], that only residues T, G, and Q, at positions 2, 23, and 26, respectively, are strictly conserved. We have also observed that proline is restricted to positions 22 and 24. Such conserved structural features could provide a framework (or possibly a limited number of frameworks) for all HVR configurations [see Lesniewski et al., 1993]. These observations on the limits to HVR variability indicate that there are considerable constraints on the range of HVR sequences that allow the virus to be viable possibly by constituting potential ligands for the target cell [see also Shimizu et al., 1994].

The reactivity of the panel samples with the HVR peptides was notable in several respects: first, that there is a marked contrast between the relatively low numbers of plasma samples recognising most of the peptides from both the E1 protein (4/45, except peptide E1.10) and the non-HVR sections of E2 (4/45) and the higher number (19/45) that recognised the HVR derived peptides (Tables I, II); second, that such a high proportion of them should have any reaction with such a limited number of HVR sequences essentially chosen at random from amongst the large number that have been reported. This result was not predictable since every HCV-infected blood sample that has been analysed contains unique HVR sequence variants [Wolfs et al., 1990; Kato et al., 1992a,b, 1994; Higashi et al., 1993; Shimizu et al., 1994], and only residues at three positions are strictly conserved. Our data appeared inconsistent with some earlier reports that individual peptides elicit antibodies that have no reaction with other HVR peptides [Weiner et al., 1992; Kato et al., 1993; Sekiya et al., 1994] and that there is no recognition of selected HVR peptides by some patients [Ching et al., 1992], although this latter result might be due to the limited numbers of peptides (1) and patients (3) analysed. The cross-reactivity reported by Lesniewski et al. may have been due to their use of peptides that contained a significant length of the HVR-contiguous conserved section of E2 protein. More recently, Zibert et al. [1995] and Scarselli et al. [1995] have shown that a significant proportion of HCV-infected patients reacted with HVR 1 peptides. Our data thus agree with and extend these latter observations.

A third feature of the data is the unexpectedly wide cross-reactivity of some of the plasma samples which reacted with several HVR peptides that differed considerably in sequence even when using a limited number of peptides, and many of the individual peptides were recognised by more than one plasma sample. Thus 40% of the plasma samples recognised at least two dissimilar peptide sequences, and 33.3% of the panel recognised three or more; two samples reacted with seven different sequences (Fig. 1). However, there was no correlation between the cross-reactivity of samples and the homology of the peptides recognised. In a total of 66 combinations, 34 involve peptides that are less than 50% homologous, of which 22 (65%) are recognised on 57 occasions (mean Abs/C-O, 2.6): 32 involve peptides that are greater than 50% homologous, of which 19 (59%) are recognised on 48 occasions (mean Abs/C-O, 2.5). The degree of cross-reactivity was not related to the level of antibodies produced since there was no correlation between either the mean Abs/C-O for any specific donor or the number of core peptides or HVR peptides with which that donor reacted (data not shown). It also seems unlikely that, by chance, the panel plasma samples would contain virus with the HVR sequences that were chosen since the potential number of amino acid combinations for a 15-mer peptide is so high. The diversity of risk factors and of genotypes suggests strongly that panel members have not been infected from an identical source of HCV. It is also likely that the circulating viral populations in the panel contained multiple quasispecies.

One possible reason for these three features of the results may be that the multiple quasispecies that exist at any time point in a single infected patient can change progressively either by continuous mutation or by selective over-expression of particular single variants [Kurosaki et al., 1994]. Thus, a wide range of anti-HVR antibodies might be elicited and accumulate in a patient over time with a consequent considerable broadening in specificity. This hypothesis would also be compatible with the observation that a high proportion of the plasma samples reacted with the de novo peptides. The conserved residues, G and Q at positions 23 and 26, were maintained throughout these sequences, and proline was restricted to positions 22 and 24; apparently sufficient sequence similarity was present to mimic some epitopes of peptides that have not been reported in patients (Table II).

However such mimicry was inexact. Thus, the percent-reactivity for each published peptide sequence was relatively unaffected by changing the residue position through which it was coupled to BSA. In contrast there was a marked decrease in the reactivities of all the de novo peptides that were coupled at the C-terminal position except the peptide pair HV11/19. This latter peptide sequence is identical with the published sequence of strain HCV-JH except for a change in the terminal two residues (KI in HCV-JH). HV11/19 therefore is substantially a natural sequence and follows, noticeably, the coupling position reactivity pattern of the published sequences. The reason for this observation is unclear but may indicate that there is a recognisable epitope at both ends of the natural peptides, as indicated by previous work [Weiner et al., 1992; Kato et al., 1994], but only one in the de novo peptides, which is C-terminal, and which may be disrupted by the coupling. The conserved sequences at positions 23 and 26 were ineffective in preserving the epitopic conformation of the C-terminally coupled de novo peptides. This sup-

ports the proposal that there are considerable constraints on natural mutations in the HVR 1 [Shimizu et al., 1994]. It is clearly of value to control the presentation in the ELISA of peptide ligands through a specific coupling to carrier protein.

A second possible explanation of the results is that there are individual antibody species that can each recognise numerous different short synthetic peptides, each of which can form a similar recognisable epitopic structure that is related in conformation to the eliciting epitope. This effective structural similarity between groups of peptides could be imposed by the strict conservation of residues at HVR residue positions 2, 23, and 26. Our finding that at certain other positions there are constraints on which amino acid residue is allowed for peptide recognition is consistent with this proposal. Moreover, it is possible to foresee a limited number of distinct epitopic structures, each having a different basic framework that is formed by both the conserved residue positions and differing sets of residues at the positions that are of constrained variability. Each distinct epitopic structure could be formed by any one of many different HVR-derived peptides providing they had the necessary pattern of essential residues to form the basic framework. Antibodies that could recognise peptides that were able to form one distinct epitopic structure would not necessarily be able to recognise sequences that could form others. However, it is known that some myeloma proteins can recognise a variety of structurally unrelated haptens [Richards et al., 1975], and therefore it is possible that a single antibody species could react with more than one distinct epitopic structure.

There is a third possibility that the short HVR peptide sequence variants may have sufficient flexibility, even when coupled to a carrier, that an individual unique sequence may be able to form more than one distinctly, recognisable epitopic conformation. This could occur either by one epitope forming exclusively at any one time (and others at other times) or by separate epitopes being formed at the same time by different sections of the same sequence, thus increasing the potential for individual sequences to be recognised by any one antibody species elicited with a single whole envelope protein. These three proposals, which could occur in combination, would make more likely the finding of reactivity when testing seropositive plasma with a limited selection of HVR representative peptides, in particular if there is a relatively low limit on the number of HVR sequences that are allowed for viral viability. The second and third hypotheses suggest the possibility a specific peptide sequence (or a very limited number of sequences) that might be able to elicit a widely cross-reactive antibody which could, if HVR is confirmed to be a neutralisation site essential to HCV replication, have a substantial role in vaccines.

In summary the data presented indicate the following possibilities: (1) A pool of anti-HVR antibodies which has a widespread specificity can accumulate in a patient; (2) there are constraints in vivo on the epitope configuration that the HVR peptides can adopt; (3) any (or one) HVR sequence in vivo may elicit antibodies that recognise many more sequences in vitro than the original challenging sequence. It is clear that our data give additional support to the proposal [Shimizu et al., 1994] that information on the limits of the mutability of the HVR and the resulting antibody specificities would be important in considering the use of HCV peptides for vaccine.

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